Exhibit 8

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Naturally formed or artificially reconstituted non-covalent alpha2-macroglobulin-peptide complexes elicit CD91-dependent cellular immunity

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Abstract

Immunization of mice with *in vitro* reconstituted alpha2-macroglobulin-peptide complexes primes peptide-specific CTL responses. We show here using the H-Y antigenic system that naturally produced, immunogenic alpha2-macroglobulin-peptide complexes can be isolated from the sera of normal male mice. As an application of these ideas to cancer immunity, we show that the immunity evoked by alpha2-macroglobulin-peptide complexes reconstituted *in vitro* is effective in prophylaxis against tumors. Furthermore, complex peptide mixtures isolated from tumor lysates can be reconstituted non-covalently with alpha2-macroglobulin and such complexes elicit potent protective tumor immunity. This approach circumvents the need for prior knowledge of the identity of the immunogenic peptides. The heat shock protein/alpha2-macroglobulin receptor CD91 is shown to be involved in the ability of heat shock proteins or alpha2-macroglobulin to elicit an anti-tumor immune response.

Introduction

Heat shock protein (HSP) preparations are immunogenic (1). The specific immunity obtained upon immunization with HSPs is derived from the peptides that they chaperone endogenously; thus HSPs purified from a tumor cell or from a virus-infected cell will be associated with tumor antigens (2) or viral antigens (3) respectively. HSPs also interact with antigen presenting cells (APCs). We and others have shown that this interaction leads to several events: (i) the release of pro-inflammatory cytokines such as IL-1beta, IL-12, GM-CSF and TNF-alpha (4), (ii) the maturation of DCs as measured by an upregulation of MHC II and B7 (4, 5) and DC migration (6), and (iii) the re-presentation of the HSP-chaperoned peptides on MHC I and MHC II molecules (7, 8). While all these events occur through receptors for signaling and internalization, only the receptor for internalization has been defined (9). The CD91 receptor has been shown to be responsible for the internalization of gp96, hsp70, hsp90 and CRT, an event in the preliminary stages of re-presentation of the chaperoned peptides (9, 10). CD91 thus provides a gateway through which HSPs are able to initiate an antigen-specific immune response. The well-

studied protease inhibitor alpha2-macroglobulin (alpha2M) is another ligand for CD91 (11, 12, 13). Like HSPs, alpha2M is also a chaperone and acts via a mechanism whereby it neutralizes proteases without necessarily destroying the proteolytic activity of the protease. Encapsulation of proteases is largely through a covalent interaction although some instances of non-covalent binding of other proteins by alpha2M have been reported (14, 15). Previously it has been shown that (i) incubation of alpha2M-peptide complexes with APCs leads to the presentation of peptides on MHC I molecules (16), and (ii) immunization of mice with alpha2M-substrate complexes primes CTLs (16) and generates antibody responses (17, 18).

In this report we investigate whether immunogenic alpha2M-peptide complexes can protect mice from a tumor challenge and test the dependence of this response, as well as the response induced with HSPs, on CD91. We also examine the nature of the interaction of peptides with alpha2M in the immunogenic complex and show that these immunogenic complexes can be purified intact from serum without *in vitro* manipulation.

Results

Generation of non-covalent alpha2M-peptide complexes

As previously shown (16), heating of alpha2M for short periods in the presence of peptide leads to an association of alpha2M with the peptide. In view of the extensive literature on the binding of a variety of substrates to alpha2M (for review see 19), we examined the nature of the alpha2M-peptide interaction in the complexes generated by heat treatment. alpha2M complexed with FITC-labeled peptides was treated with PBS (negative control) or was subjected to denaturing conditions (2% SDS or 2% SDS, 0.1 M 2-mercaptoethanol) under which non-covalent bonds are disrupted. The treatments were carried out for 1 h and the products analyzed by SDS-PAGE and immunoblotting with an anti-FITC monoclonal antibody. The native homo-tetrameric (720 kDa) alpha2M dissociates into dimers (360 kDa) with SDS treatment (held by disulphide bonds) or monomers (180 kDa) with SDS and 2-mercaptoethanol treatment as shown previously (20) and in the schematic diagram in Figure 1. Figure 1 also shows that complexes treated with SDS (lanes 3 and 4) or with SDS and 2-mercaptoethanol (lanes 5 and 6) did not blot with anti-FITC antibodies (lanes 4 and 6) indicating that peptides did not remain bound to alpha2M under either of these conditions. PBS-treated complexes (lanes 1 and 2) were applied to SDS-PAGE in non-denaturing buffer (even though the gel contains SDS) and were immunoblotted. These complexes were recognized by anti-FITC antibody (lane 2) indicating that the peptides remained bound under conditions of electrophoresis. Since SDS cannot disrupt covalent bonds, the results demonstrate that the interaction of alpha2M with peptides in complexes generated by heat treatment is non-covalent.

Anti-tumor immunity elicited by alpha2M-peptide complexes

Gp96-peptide or alpha2M-peptide complexes were reconstituted as described in Materials and methods. C57BL/6 mice were immunized intradermally with alpha2M-ova20 complexes twice, at a one week interval. The immunization regimen is outlined in Figure 2A. The mice were challenged with 100 000 live B16-F10-OVA (ovalbumin-expressing) tumor cells one week after the last immunization. As a positive control mice were immunized with gp96-ova20 complexes. Mice were also immunized with alpha2M or gp96 without peptide or with PBS. Tumor growth (and survival) was monitored. As shown in Figure 2B, tumor growth was significantly delayed or no tumor growth was observed in mice immunized with alpha2M-ova20 complexes ($P<0.05$) or gp96-ova20 complexes ($P<0.05$). Indeed 5/5 mice (alpha2M-ova20 group) and 4/5 mice (gp96-ova20) survived past day 22. Mice immunized with PBS, alpha2M or gp96 grew large tumors and few mice (1/5, 1/5, 2/5 respectively) survived past day 22 after tumor challenge (some had to be sacrificed).

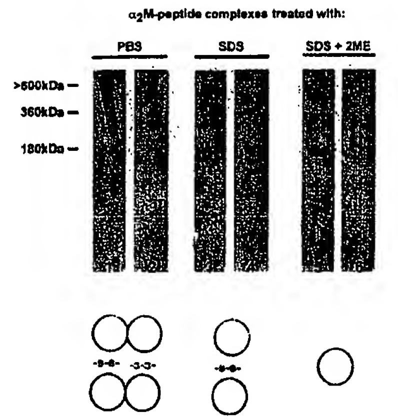

Figure 1. Non-covalent bonds are present in the alpha2M-peptide complexes. alpha2M was complexed to FITC-labeled peptide at a 1:50 molar ratio as described in Materials and methods. alpha2M-peptide complexes treated with SDS (lanes 3 and 4), with SDS and 2-ME (lanes 5 and 6) or untreated (lanes 1 and 2) were analyzed by SDS-PAGE (left panels for each treatment; lanes 1, 3 and 5) and immunoblotting with anti-FITC antibody (right panels; lanes 2, 4 and 6). Dissociation of the native alpha2M protein (720 kDa) by SDS denaturation produces a dimeric subunit (360 kDa). The disulphide bonds holding the dimer together can be cleaved using 2-ME. The monomeric subunits are 180 kDa.

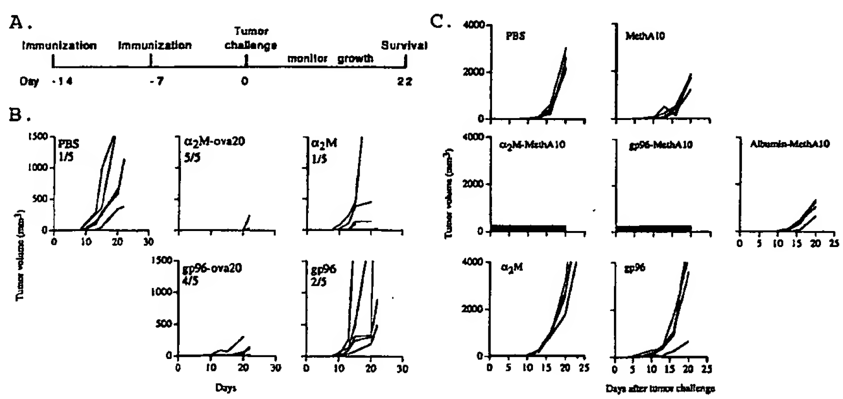

Figure 2. Non-covalent alpha2M-peptide complexes are immunogenic and can protect mice from a tumor challenge. (A) Protein-peptide complexes were prepared as described and injected into mice intradermally on days -14 and -7. Mice were challenged with live tumor cells on day 0 and tumor growth was monitored. (B) alpha2M or gp96 was complexed with synthetic ova20 peptide containing the CTL epitope of OVA. Mice were challenged with B16-F10 transfected with OVA. The fractions in the top left corner of each panel correspond to the number of mice surviving past day 22. (C) alpha2M, gp96 or albumin was complexed to a preparation of Meth A tumor-derived peptides (MethA10) as described in Materials and methods. Mice were challenged with Meth A. In B and C control mice were injected with uncomplexed alpha2M or gp96, with peptide alone or with PBS.

A second tumor system, the methylcholanthrene-induced fibrosarcoma (Meth A) tumor model, was used. The antigenic MHC I epitope(s) of this tumor is(are) unknown. Meth A cell lysates were treated with ATP or TFA and the fraction of peptides smaller than 10 kDa was collected as described in Materials and methods. The peptide preparation (called MethA10) was complexed to alpha2M or gp96 as described above. BALB/c mice were immunized with alpha2M or gp96, either uncomplexed or complexed with MethA10. BALB/c mice were also immunized with albumin-MethA10 or PBS as negative controls. The mice were immunized twice, at a one week interval (Figure 2A). All mice were challenged intradermally with 100 000 live Meth A cells one week after the last immunization and tumor growth was monitored. Figure 2C shows significant tumor protection in mice immunized with alpha2M-MethA10 (P<0.05) or gp96-MethA10 (P<0.05) complexes but not in mice immunized with alpha2M, gp96, albumin-MethA10 or PBS.

The immunogenicity of alpha2M- and gp96-peptide complexes is dependent on CD91

alpha2M and gp96 both interact independently with the CD91 receptor. As previously shown, this interaction is necessary for the cross-presentation *in vitro* of the chaperoned peptides for both ligands (9, 16). We tested the hypothesis that this interaction would also be essential to generate (anti-tumor) immune responses *in vivo* using gp96- or alpha2M-peptide complexes as immunogens. The B16-F10-OVA tumor model was used. Mice were immunized as in Figure 2A with alpha2M-ova20 or gp96-ova20 complexes mixed with an anti-CD91 antibody. This was compared to mice immunized with complexes mixed with an isotype control antibody. Additional antibody was given each day for 2 days after immunization at the same site. Figure 3 shows that the anti-CD91 antibody is effective at blocking the anti-tumor immune response generated by alpha2M (P<0.005) or gp96 (P<0.005). Control isotype antibody did not show results dissimilar from Figure 2B where no antibody was given. These results directly implicate CD91 (as a molecule or the expressing cells) as being a central player in eliciting immune responses with gp96 or alpha2M.

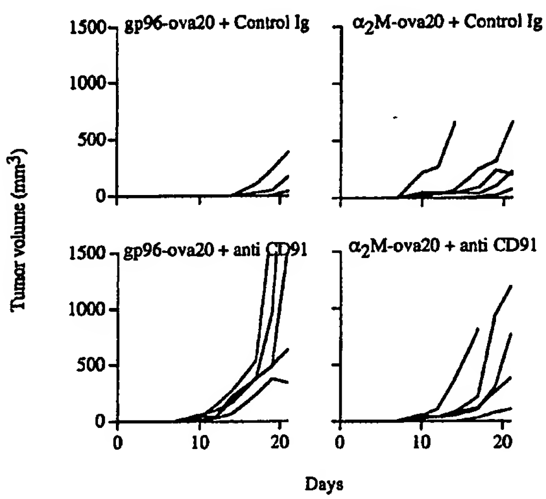

Figure 3. Tumor immunity is dependent on CD91. Mice were immunized as in Figure 2A together with 100 µg of anti-CD91 antibody or control rabbit Ig. Further antibody was given on days -13, -12, -6 and -5. The B16-F10-OVA tumor challenge was given on day 0 and tumor growth was monitored.

alpha2M is associated with antigens *in vivo*

The alpha2M-antigen complexes used for the tumor rejection assays (Figure 2) and for studies elsewhere (16, 17, 18) were generated artificially by thermal manipulation. We sought to determine if alpha2M was associated with antigenic peptides physiologically. The question was addressed using the male H-Y antigen system as the model. alpha2M was purified from the sera of male C57BL/6 mice as described in Materials and methods and gp96 was purified from the livers of male mice as previously described (9). Female mice (which do not express the H-Y antigen) were immunized with male serum-derived alpha2M or male liver-derived gp96 twice, at a one week interval. Spleens from the immunized female mice were removed on the third week and stimulated twice *in vitro* with irradiated male spleen cells. Cytotoxic T lymphocyte (CTL) assays were performed 4 days after the last *in vitro* restimulation using male and female (negative control) spleen cells as targets. Figure 4 shows that CTLs from the spleens of two independently immunized mice lyse male, but not female, spleen target cells. There was no lysis of either target by spleen cells from female mice immunized with PBS. alpha2M purified from serum may thus be deduced to contain immunogenic H-Y antigenic peptides without requiring any treatment, incubations or addition of antigen.

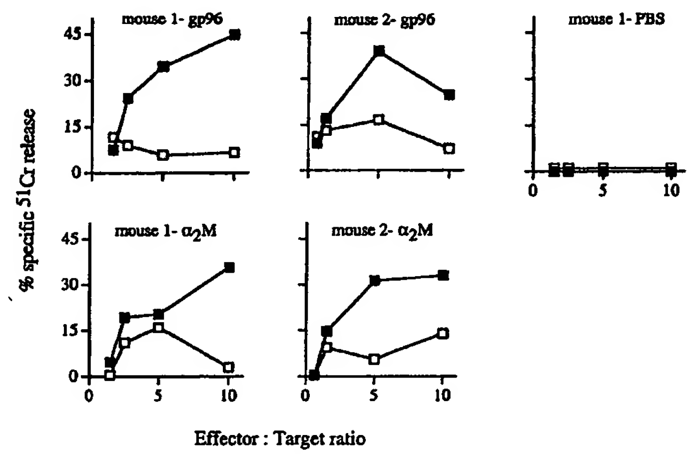

Figure 4. Endogenous alpha2M-peptide complexes are immunogenic. alpha2M was purified from the sera of male C57BL/6 mice as described. Syngeneic female mice were immunized twice with 7 µg of protein or PBS. Spleens were removed from immunized mice and cytotoxic assays were performed after two restimulations against male (filled squares) and female (open squares) target cells.

Discussion

The ability of HSP-peptide complexes to evoke tumor immunity has been demonstrated for many years. The examination of this phenomenon has revealed that the interaction of HSPs with receptors on antigen presenting cells is a necessary event for the internalization of the HSP with the peptide and the ensuing presentation of the peptide on MHC molecules (7, 9). CD91 has been identified as the receptor necessary for the internalization of gp96, hsp90, hsp70 and calreticulin (10). CD91, also known as LRP or alpha2MR, was originally identified as a receptor for alpha2M. We and others have shown that preparations of alpha2M carrying an antigen are immunogenic with respect to the antigen, as measured by the induction of CTL responses and antibodies. In this report we demonstrate the anti-tumor efficacy of alpha2M-peptide complexes. The immunogenicity of alpha2M-peptide complexes was not qualitatively or quantitatively different from gp96-peptide complexes. Mice immunized

with either complex mounted comparable anti-tumor responses leading to protection against a tumor challenge. Both gp96 and alpha2M bind to CD91 and are internalized by this receptor. We extended our studies to test the role of CD91 in generating an anti-tumor response. CD91-deficient mice are embryonic lethal (21). We were therefore limited to co-injecting anti-CD91 antibody along with gp96- or alpha2M-peptide complexes. A lack of anti-tumor immunity in mice given anti-CD91 antibody with either complex was observed. This observation can be explained by at least two mechanisms that are not mutually exclusive: (i) Anti-CD91 antibody blocks the interaction of alpha2M or gp96 with CD91. We have previously shown that this antibody blocks cross-presentation of antigens chaperoned by gp96 and alpha2M (10). APCs expressing CD91 would therefore not be able to re-present immunogenic peptides, chaperoned by gp96 or alpha2M, to T cells *in vivo*. (ii) Anti-CD91 antibody causes the depletion of APCs expressing CD91. This explanation seems unlikely since the antibodies are given at the same time with alpha2M or gp96. Effective depletion may be possible if the antibody was given 1-2 days prior to the immunization. It would be of interest to deplete local CD91-expressing cells before administration of the complexes. It is our view that the antibodies block the interaction of CD91 with the adjuvant, hence preventing uptake.

The experiments involving immunization against tumors shown here suggest a novel approach to immunotherapy of cancers, whereby one may generate an array of peptides including self and antigenic peptides from the total tumor and complex them to an HSP or alpha2M, or for that matter other CD91 ligands. Such complexes are effective in prophylaxis as shown here. Their utility in immunotherapy of pre-existing disease is currently being tested. This paradigm may be extended to pathogenic infections as well.

Abbreviations

alpha2M, alpha2-macroglobulin; HSP, heat shock protein; Meth A, methylcholanthrene-induced fibrosarcoma

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Materials and methods

Protein purification

For alpha2M purification, serum from mice was diluted 1:1 with 0.04 M Tris pH 7.6, 0.15 M NaCl and applied to a 65 ml Sephacryl S 300R (Sigma) column equilibrated and eluted with the same buffer. alpha2M-positive fractions were determined by a dot blot and the buffer changed to a 0.01 M sodium phosphate buffer pH 7.5. The protein was applied to a concanavalin A sepharose column. Bound protein was eluted with 0.2 M methylmannose pyranoside and applied to a DEAE column equilibrated with 0.05 M sodium acetate buffer. alpha2M was eluted in a pure form, as analyzed by SDS-PAGE and immunoblotting with 0.13 M sodium acetate. Some experiments were carried out with alpha2M purchased from Sigma.

Tumor rejection assays

BALB/c or C57BL/6 mice were obtained from Jackson Labs (Bar Harbor, ME) and used at 6-8 weeks of age. All immunizations were done intradermally in 100 µl PBS. Two immunizations were given at a one week interval. Seven micrograms of alpha2M and 1 µg of gp96 were used per injection either as a complex or alone. Live tumor cells (100 000) were washed free of culture medium, resuspended in PBS and injected intradermally one week after the last immunization. For CD91 blocking studies, anti-CD91 antibody (described in 9) or control rabbit IgG was mixed with the complex prior to injection. 100 µg of antibody was given per injection. A further 100 µg of antibody was injected at the same site one and two days after (days -13, -12, -6, and -5). Tumors were measured in 2 dimensions. Half of the average was used as the radius of the tumor to calculate the tumor volume. *P* values were determined using single-classification analysis of variance (ANOVA).

Generation of protein-peptide complexes

Cell lysate was obtained from live Meth A cells by Dounce homogenization followed by ultracentrifugation at 100 000 g. The supernatant was treated with 0.1% trifluoroacetic acid and 3 mM ATP for 10 h followed by centrifugation in a Centricon (Millipore) with a 10 kDa cut-off membrane. Peptides smaller than 10 kDa (MethA10) were used for complexing. The ova20-mer peptide (NH_2-SGLEQLE**SIINFEKL**TEWTS-COOH) was synthesized by Genemed (CA) to >95% purity. The protein was heated to 50°C in the presence of 50 molar excess of ova20 or MethA10. The complexes were placed at room temperature for 30 min and then placed on ice. Free, uncomplexed peptide was removed using a Centricon 50 (Millipore). Complexes thus made were used for immunizations.

Complexes that were made for further analysis (Figure 1) were prepared in a similar manner except that the ova20 was labeled with FITC prior to complexing. FITC labeling was performed using the FITC-1 kit from Sigma. After complexing and removing free peptides, the complexes were divided into the appropriate number of tubes and treated with 2% SDS alone or with 2% SDS and 0.2 M 2-mercaptoethanol. Complexes treated or not were analyzed by 7% SDS-PAGE containing 0.01% SDS and immunoblotted using an anti-FITC monoclonal antibody (Sigma).

Generation of anti-H-Y cytotoxic T cells

Female mice were injected subcutaneously with male-derived gp96, alpha2M or PBS. Spleens from immunized female mice were placed in RPMI supplemented with 5% FCS (complete RPMI) for one week with irradiated male spleen cells. Every 7 days thereafter spleen cells were re-stimulated with irradiated male spleen cells and rat concanavalin A supernatant. CTL assays were performed on day 4 after restimulation using ^{51}Cr-labeled male and female spleen cells as targets.

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